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## Functional properties of sodium and calcium caseinate antimicrobial active films containing carvacrol

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### Abstract:

Active edible films were prepared by adding carvacrol into sodium caseinate (SC) and calcium caseinate (CC) matrices plasticized with two different glycerol concentrations (25 and 35 wt%) prepared by solvent casting. Functional characterization of these bio-films was carried out by determination of some of their physico-chemical properties, such as colour, transparency, oxygen barrier, wettability, dye permeation properties and antibacterial effectiveness against Gram negative and Gram positive bacteria. All films exhibited good performance in terms of optical properties in the CIELab space showing high transparency. Carvacrol was able to reduce CC oxygen permeability and slightly increased the surface hydrophobicity. Dye diffusion experiments were performed to evaluate permeation properties. The diffusion of dye through films revealed that SC was more permeable than CC. The agar diffusion method was used for the evaluation of the films antimicrobial effectiveness against *Escherichia coli* and *Staphylococcus aureus*. Both SC and CC edible films with carvacrol showed inhibitory effects on both bacteria.

**Key words:** active food packaging; edible films; carvacrol; caseinates; dye diffusion; antimicrobial properties.

## 1. Introduction

The use of bio-based and biodegradable materials in food packaging is currently increasing to reduce the use of thermoplastics obtained from non-renewable resources. In this sense, caseinates have been suggested as raw materials for food packaging films since proteins show some advantageous properties, such as their ability to form networks, plasticity and elasticity (Pereda et al., 2008) as well as good barrier to oxygen, carbon dioxide and aromas (Caprioli et al., 2009). It is known that oxygen plays an important role in food degradation, as it is involved in many undesirable reactions including microorganisms growth, enzymatic browning, vitamin loss and lipid oxidation (Caprioli et al., 2009). Therefore the use of caseinates could be considered an alternative to get high protection to oxygen in important food processing technologies, such as modified atmosphere packaging. Moreover, caseinates have been reported as good environmentally-friendly carriers of antimicrobial compounds (Kristo et al., 2008).

Research in active packaging is a current tendency in food technology since these systems take advantage of their interaction with food, having an important effect on the shelf-life extension (Arrieta et al., 2013b). Antimicrobial food packaging is one of the most promising approaches, since active films help to minimize the contamination of food products by microorganisms during storage, transportation and handling (Quintavalla and Vicini, 2002), while controlling spoilage and pathogenic microorganisms (Moreira et al., 2011). The design of efficient antimicrobial packaging systems requires the complete study of the way to introduce generally volatile antimicrobial compounds in films. Considerable interest in extracts and essential oils from aromatic plants with antimicrobial activity has raised to control pathogenic microorganisms growth and toxin formation in food (Viuda-Martos et al., 2007). Essential oils also have a large acceptance by consumers (Rubilar et al., 2013). For instance, carvacrol, a major constituent of oregano essential oil, was found to be an efficient antimicrobial agent (Ben Arfa et al., 2006; Burt, 2004; Guarda et al., 2011). It has been demonstrated that carvacrol is highly active against strains of *Staphylococcus aureus* and *Escherichia coli* (Chalier et al., 2007), including the pathogen *E. coli* O157:H7 (Du et al., 2008; Lim et al., 2010). However, the addition of active agents into polymer matrices may affect their transparency and other optical properties (Quintavalla and Vicini, 2002). This could be a negative issue by the increasing demand for transparent and colourless food packaging materials. Another apparent drawback from the use of carvacrol in active packaging systems is the slight characteristic oregano odor (Arrieta et al., 2013b), and this should be taken into

account when considering the final application of these active systems. It is known that antimicrobial compounds are commonly incorporated to meat products to extend their shelf-life (Ruiz-Navajas et al., 2013). Therefore, the use of carvacrol as active agent could provide good antimicrobial protection without the need of direct incorporation of a food additive onto the meat surface, besides providing acceptable organoleptic properties.

It has been demonstrated that carvacrol shows high compatibility with caseinates and this could help to increase the material poor tensile properties (Arrieta et al., 2013b). But, the inherent brittleness of caseinates should be overcome by the addition of plasticizers to get the required flexibility for films manufacturing. Glycerol, a major by-product in biodiesel production, is one of the most important natural plasticizers. Moreover, the use of glycerol as an additive for bio-based polymers increase its value from a low-grade by-product to a useful compound (Xiu et al., 2011).

The main objective of this research is the evaluation of functional properties of high transparent antimicrobial bio-films based on plasticized sodium and calcium caseinates with carvacrol and their suitability for food active packaging applications, particularly for the meat industry. For this purpose, films were tested against two indicator bacteria, *E. coli* (Gram negative) and *S. aureus* (Gram positive) to evaluate their antibacterial efficiency. Their optical properties were also determined by using the CIELab space coordinates. Finally, oxygen barrier properties, wettability and dye diffusion conditions were also evaluated for all formulations.

## **2. Methods and techniques**

### **2.1. Materials**

Sodium caseinate (SC) and calcium caseinate (CC) powders were kindly supplied by Ferrer Alimentación S.A (Barcelona, Spain). Carvacrol (98% purity) (CRV) was obtained from Sigma Aldrich (Móstoles, Spain). Anhydrous Glycerol (99.5% purity) was purchased from Fluka (Madrid, Spain). The solvent for casting processing was triple-distilled and deionized water in all cases.

### **2.2. Film preparation**

The casting solution was prepared by following the method already developed and reported elsewhere (Arrieta et al., 2013b). In summary, 5 wt% of caseinate, either SC or CC, and the amount of glycerol required to obtain protein:glycerol 1:0.25, 1:0.35 ratios (in weight) were prepared in deionized water under continuous stirring. These solutions were heated at 65 °C with magnetic stirring for 10 minutes at 1100 rpm and

cooled at room temperature. Carvacrol was further added to get a constant 1:0.1 protein:carvacrol ratio (in weight) and solutions were stirred for 3 additional min at 1100 rpm. All solutions were degasified in ultrasound bath at room temperature and films were further prepared by casting. 30.0 mL of film-forming solutions were put into 15 cm diameter polyethylene Petri dishes (Distrilab S.L., Cartagena, Spain) and dried at  $25 \pm 2$  °C and 50% constant relative humidity (RH) in a Dycometal-CM81 climatic testing chamber (Barcelona, Spain) for 48 h. Glycerol and carvacrol concentrations used in this study were previously optimized and reported (Arrieta et al., 2013b). Thickness of the obtained films was measured with a Digimatic Micrometer Series 293 MDC-Lite (Mitutoyo, Tokyo, Japan) at ten random positions over the film surface (Table 1). Films were stored for not longer than 5 days at 4 °C before testing.

### 2.3. Film colour

Film colour properties were evaluated by using a KONICA CM-3600d COLORFLEX-DIFF2, HunterLab colourimeter, (Hunter Associates Laboratory, Inc, Reston, VA, USA). According to the CIELab colour space, results were expressed as colour coordinates  $L^*$ ,  $a^*$ ,  $b^*$  (Lightness, red-green and yellow-blue respectively). Measurements were taken in five random positions around the films surface. Total colour differences ( $\Delta E$ ) induced by the incorporation of the active agent were calculated with respect to SC or CC control films (films without active agent).

### 2.4. Oxygen transmission rate

Oxygen transmission through films was measured by using an oxygen permeation analyzer, Systech Instruments-Model 8500 (Metrotec S.A, Spain). Films (14 cm diameter circles) were clamped in the diffusion chamber at  $25 \pm 2$  °C. Pure oxygen (99.9% purity) was introduced into the upper half of the sample chamber while pure nitrogen (99.9% purity) was injected into the lower half where an oxygen sensor was placed. Films were equilibrated at  $25 \pm 2$  °C and 50% constant RH for 24 h before testing. Tests for each film formulation were performed in triplicate and were expressed as oxygen transmission rate per film thickness (OTR.e).

### 2.5. Antimicrobial activity

SC and CC films with carvacrol were individually tested against *Staphylococcus aureus* ATCC 25922 and *Escherichia coli* ATCC 25923. Both bacterial cultures were supplied by the Spanish Type Culture Collection (CECT, University of Valencia, Spain).

The agar diffusion method was used to determine the antibacterial performance of films against *S. aureus* and *E. coli* by following the methodology recommended by

NCCLS (National Committee for Clinical Laboratory Standards, 1990). Suspensions ( $0.1 \text{ mL}$  of  $10^6 \text{ CFU mL}^{-1}$ ) of each microorganism were spread on solid medium plates (Nutrient Agar, Insulab, Spain) and were incubated at  $37^\circ\text{C}$  for 48 h to obtain isolated colonies. 4 or 5 well isolated colonies of each microorganism were then put in contact with tryptone soy agar (TSA, Insulab, Spain) and incubated at  $37^\circ\text{C}$  for approximately 5 h to achieve a turbidity of 0.5 (McFarland scale). Two successive dilutions ( $1 \text{ mL}$  solution and  $9 \text{ mL}$  deionized water) were carried out to reach a final count of  $10^6 \text{ CFU mL}^{-1}$ .  $0.1 \text{ mL}$  of each inoculum suspensions were put in solid medium plates (Mueller Hinton Agar, Insulab, Spain) in contact with  $100 \text{ mm}^2$  films and incubated at  $37^\circ\text{C}$  for 18 h. The antimicrobial potential of films was quantitatively assessed by following the methodology reported by Seydim and Sarikus (Seydim and Sarikus, 2006). The “inhibition zone” was calculated by subtraction of the whole film surface and the area with no microorganisms growth. All tests were performed in triplicate in each plate and in duplicate between plates. Films with no carvacrol were treated by using the same protocol and were used as control.

## 2.6. Wettability

The surface hydrophobicity of films was studied by measuring the water contact angle. Analyses were carried out by using an EasyDrop Standard goniometer FM140 (KRÜSS GmbH, Hamburg, Germany) equipped with a camera and analysis software (Drop Shape Analysis SW21; DSA1). The contact angle was measured by randomly adding with a syringe some drops of distilled water ( $\approx 2 \mu\text{L}$ ) to the surface film at room temperature (Arrieta et al., 2013a). Prior to testing films were equilibrated at  $25 \pm 2^\circ\text{C}$  and 50% constant RH for 24 h. Ten contact angle measurements were carried out for each drop and average values of six drops were calculated and reported.

## 2.7. Permeation studies

Permeability of dyes through films was measured using a previously reported device (Lobo et al., 2001; Valente et al., 2000) consisting on two  $200 \text{ mL}$  (V) compartments, A for the donor and B for the receptor, interconnected through an opening area (A)  $0.659 \text{ cm}^2$ . Films were sealed with silicone and placed between these two compartments. Solutions in both compartments were stirred at ca. 200 rpm. Crystal violet (Fluka,  $M = 407.99 \text{ g mol}^{-1}$ ) was dissolved in methanol (Fluka, 99.5% purity) and this methanolic solution (CV) was chosen as the testing dye (Papancea et al., 2010a). It was observed that during the sorption process films changed from colourless to blue but they remained stable during the sorption/desorption process. A Velp Scientifica thermostatic bath was used to keep the solutions temperature at  $25.0 \pm 0.1^\circ\text{C}$ . The

dye flux through films was quantified by measuring the absorbance of the dye solution, in compartment B, at 586 nm (UV-VIS 2450 CE 230V spectrometer SHIMADZU Corporation, Kyoto Japan). The obtained absorbance values were converted into concentration values by considering a molar extinction coefficient ( $\epsilon$ ) equal to 34891 M<sup>-1</sup> cm<sup>-1</sup> at the cited wavelength. The  $\epsilon$  value was calculated by measuring the absorbance, at 586 nm, of CV standard solutions in the concentration range: 8 x 10<sup>-7</sup> to 1.5 x 10<sup>-3</sup> M. Permeation was monitored in a daily basis from the solution in the B compartment for 2 weeks. Figure 1 shows a representative experiment for the permeation of CV through a CC film as a function of time.

The steady-state flux,  $J$ , of the dye was calculated from

$$J = (V / A)(dc / dt) \quad (1)$$

Describing the permeability of the permeant in terms of a simple diffusion process

$$\frac{\partial C}{\partial x} = \frac{\partial}{\partial x} (D_F \frac{\partial C}{\partial x}) \quad (2)$$

with the boundary and initial conditions  $C(0,t) = Kc$ ;  $C(l,t) = 0$  and  $C(x,0) = 0$  (where  $C$  and  $c$  are the initial concentrations inside the film and in aqueous solutions,  $x$  the space coordinate,  $t$  the time and  $K$  the partition coefficient), resulting in the simple formulae for calculation of the permeability,  $P$ , and diffusion,  $D_F$ , coefficients (Valente et al., 2004):

$$P = Jl / c_{dye} \quad (3)$$

$$D_F = l^2 / (6\theta) \quad (4)$$

where  $c_{dye}$  is the concentration of the methanolic CV dye solution (1 x 10<sup>-4</sup> M) in compartment A,  $l$  is the film thickness measured after each experiment at 25 °C and  $\theta$  is the time-lag. Low dye solution concentration was chosen with the main objective to ensure a constant level before the steady state is achieved (Figure 1).

## 2.8. Statistical analysis



Experimental data were processed with the aid of OriginPro 8 software. One-way analysis of variance (ANOVA) was carried out and significant differences among formulations were recorded at  $p < 0.05$  according to Tukey's post hoc test.

### 3. Results and discussion

#### 3.1. Optical colour properties

Figure 2 shows some photographs of the obtained SC (Figure 2a) and CC (Figure 2b) films. The composition and designation of all obtained films are summarized in Table 1. Transparency is an important issue in consideration of the potential use of these films in food packaging (Fabra et al., 2009). As can be observed, plasticized caseinates kept both their transparency and optical properties after the addition of the active compound (Figure 2).

It has been indicated that the optical properties of polymer films are dependent on the nature of the additives used in their formulation (Irissin-Mangata et al., 2001). The variations in colour of all caseinate films represented by their CIELab coordinates are shown in Table 2. It was observed that lightness values ( $L$ ) showed a similar trend in films based in both caseinates, with slight increase with the glycerol addition, regardless of the CRV content. Moreover, the total colour differences ( $\Delta E$ ) were smaller than 2.0 in all cases, being this value the threshold of perceptible color difference for the human eye (Horie et al., 2012; Paravina et al., 2002), confirming the high clearness and colourless of all plasticized SC and CC films, with and without the active compound. The variation in the optical properties of SC films plasticized with glycerol was also investigated by other authors who proposed that the presence of oils in these formulations reduces their transparency (Fabra et al., 2009; Pereda et al., 2010). In fact, a slight increase in amber colour for SC films plasticized with glycerol and essential oils at concentrations up to 10 wt% was reported (Pereda et al., 2010). Fabra et al. also found that SC films containing glycerol exhibited high transparency, whereas the presence of lipid mixtures of oleic acid and beeswax increased the film opacity and darkness (Fabra et al., 2009). But this effect should be carefully taken into account since it has been reported that the addition of antimicrobial agents to polymer films might influence some of their physical properties, in particular transparency and colour (Quintavalla and Vicini, 2002). In this study it was observed that the incorporation of carvacrol to plasticized SC or CC films had no apparent influence on their transparency (Figure 2). As previously stated, positive values for the  $b^*$  coordinate are indicative of deviation towards yellow and no significant differences in this parameter were observed between SC films. Only the CC film with the highest amount of G (35 wt%) containing CRV (10 wt%) showed significant differences ( $p < 0.05$ ) in this parameter. On the other



hand, negative values obtained for  $a^*$  coordinate are indicative of deviation towards green colour. This coordinate significantly decreased ( $p > 0.05$ ) in SC films with the presence of CRV. Meanwhile, slight ( $p < 0.05$ ) differences were observed between plasticized CC with CRV and their non-active counterparts.

### 3.2. Oxygen transmission rate

Barrier properties are one of the most important issues to be considered in materials intended to be used in food packaging, since the presence of oxygen could lead to the decrease in food shelf-life and quality (Li et al., 2013). Oxygen transmission rate (OTR.e) of all SC and CC-based films was measured and results are shown in Figure 3. It was noticed that the low OTR.e values obtained for all formulations confirmed that caseinates-based films showed excellent oxygen barrier properties. It should be pointed out that these values are similar to those obtained for poly(ethylene terephthalate) (PET) films and clearly lower than those for poly(lactic acid) films with similar thickness (Martino et al., 2009). Therefore, caseinates films showed, in general, excellent barrier properties making them highly useful in the formulation of food packaging materials where barrier to oxygen is critical. However, as reported for other biopolymer matrices, significant increases in OTR.e values should be expected with the addition of plasticizers (Martino et al., 2009) due to the increase in the polymer chain mobility and allowing the transport of oxygen molecules through the polymer matrix (Arrieta et al., 2013a). It was observed that the increase in the G amount into caseinate films resulted in some slight increase in oxygen permeation in SC films, while a significant decrease in OTR.e values was noticed into CC films ( $p > 0.05$ ). This behaviour was more noticeable when CRV was added to the plasticized caseinates. The OTR.e values for SC films slightly increased while they noteworthy decreased in CC-based formulations ( $p < 0.05$ ), demonstrating the additional advantage of the CRV addition to plasticized CC matrices with very low oxygen transmission and consequently limiting food oxidation. Furthermore, these changes in oxygen barrier properties upon the addition of CRV were more intense in those formulations with the higher G concentration. Similar results were obtained by Valente et al. (Valente et al., 2007), who reported that the incorporation of calix[4]pyrrole into cellulose matrices resulted in an alteration in the composites hydrophobicity and consequently in the oxygen permeation. In a previous work we reported that the addition of G and CRV to caseinates films, changed the biopolymer morphologies by the presence of free  $-OH$  functional groups resulting in the formation of hydrogen bonds in the materials internal structure (Arrieta et al., 2013b). The electrostatic interactions between caseinates molecules and the active agent free hydroxyl groups are important and it could be

concluded that the highest interaction of the free hydroxyl groups with calcium dications could result in a higher reduction in oxygen transmission in CC films. Similar results were obtained by Tomasula et al. who reported that hydrophilic CC has in its structure a lot of available sites for binding of glycerol. Therefore, CC films show less free volume available for diffusion of oxygen and as a result they showed lower values of oxygen transmission rate than other casein based films (Tomasula et al., 2003).

### 3.3. Antimicrobial properties of films

*S. aureus* and *E. coli* cultures grew normally on agar plates with films with no CRV in their formulation, while films based on both caseinates with the addition of CRV exhibited antimicrobial activity with the presence of the inhibition zone. The CRV antimicrobial mechanism is based on the disturbance of the bacteria cytoplasmic membrane, with disruption of the proton motive force, electron flow, active transport and coagulation of cell contents. In addition, CRV is able to disintegrate the outer membrane of Gram-negative bacteria (Burt, 2004) resulting in inhibition of the microorganisms growth. The agar diffusion method simulates food wrapping, reproducing the situation when films are in direct contact with food and the possibility of release of the antimicrobial agent (Appendini and Hotchkiss, 2002; Guarda et al., 2011). CRV, was selected as this compound is classified as Generally Recognized as Safe (GRAS), being one of the main components of thyme spice, by the United States Food and Drug Administration (FDA; Lambert et al., 2001). The method used in this qualitative study is based on the diffusion of the active agent through the agar gel resulting in an inhibition zone around the film area, showing the CRV inhibitory effect against both bacteria. The antimicrobial activity of these caseinate films against *E. coli* and *S. aureus* was also confirmed by measuring the inhibition zone diameter (Figure 4). The antimicrobial efficiency of CRV against *E. coli* was previously reported in low density polyethylene (LDPE)/polyamide films (Han et al., 2007) and in wheat gluten montmorillonite coated papers (Mascheroni et al., 2011). Polypropylene with CRV films showed inhibitory effect against *S. aureus* (Ramos et al., 2012), while similar results against both, *E. coli* and *S. aureus*, were reported in films based on polyethylene-co-vinylacetate (EVA) with CRV (Nostro et al., 2012) and in methyl cellulose/montmorillonite with CRV (Tunc and Duman, 2011). In this study, sodium and calcium caseinates films with CRV showed antibacterial effectiveness against both gram positive (*S. aureus*) and gram negative bacteria (*E. coli*), such as those already reported with the additional advantage of using biopolymers, such as caseinates. Figure 4 shows that *S. aureus* was more sensitive to CRV than *E. coli*, showing larger inhibition zone, indicating that the antimicrobial sensitivity of microorganisms depends

on their chemical interaction with the active agent. These results are in accordance with those reported by other authors (Burt, 2004; Emiroglu et al., 2010) who concluded that Gram-positive microorganisms are more sensitive to oregano essential oils than Gram-negative bacteria. This result could be expected, since these microorganisms possess an outer membrane surrounding the cell wall which restricts diffusion of hydrophobic compounds through its lipophilic polysaccharide structure and consequently giving them higher resistance to the CRV activity (Burt, 2004).

On the other hand, it was observed that the *E. coli* inhibition zone was larger for SC films ( $p < 0.05$ ). As stated when discussing oxygen barrier properties, this behaviour could be due to the fact that divalent calcium cations in CC promote cross-linking with protein chains (Arrieta et al., 2013b; Fabra et al., 2010) giving a more tortuous structure to retain CRV more efficiently and consequently releasing the active agent more slowly. In general terms it can be concluded that CRV can be used as antibacterial agent in caseinates films which are good matrices with ability to release the active component.

### 3.4. Wettability

In a previous work it was concluded that caseinates plasticized with 35 wt% of glycerol showed adequate mechanical properties for films manufacturing (Arrieta et al., 2013b). In the present work we observed that these films exhibited high transparency, excellent barrier properties to oxygen and antimicrobial effect against *E. coli* and *S. aureus*. Therefore, we selected caseinates plasticized with 35 wt% of glycerol for further analysis, i.e. wettability and dye diffusion experiments.

It was already discussed that surface hydrophobicity is an important issue in this kind of materials. Therefore, water contact angle measurements were carried out to study the water absorption of these material surfaces (Arrieta et al., 2013a). It is known that the water contact angle is dependent on the cohesive and adhesive molecular forces within the water and between the water and the solid surface respectively (Hambleton et al., 2009; Pereda et al., 2011; Vogler, 1998). A contact angle ( $\theta$ ) higher than  $65^\circ$  is typical for hydrophobic surfaces, meanwhile  $\theta < 65^\circ$  is observed in hydrophilic surfaces (Hambleton et al., 2009; Vogler, 1998). Results for water contact angle in these films are reported in Table 3. In general, low values were obtained, suggesting the typical hydrophilic character of caseinates even after the plasticizer addition (Aliheidari et al., 2013; Arrieta et al., 2013b). Moreover, it has been reported that the interaction between glycerol and polymer chains could reduce the polymer-polymer interactions by increasing the chains mobility and consequently decreasing the surface hydrophobic character (Hambleton et al., 2009). It was observed that the SC film containing CRV gave higher water contact angle ( $p < 0.05$ ) than the SC film

without CRV (Table 3) showing the ability of the active agent to increase the hydrophobic performance of the SC films. This effect could be partially attributed to the fact that the presence of CRV facilitates the formation of an emulsion in the caseinate-glycerol aqueous solution. This emulsion would be dependent on the relationship between  $\alpha$  and  $\beta$  casein components adsorbed at the CRV oil droplet, as it was already observed by optical microscopy and infrared analysis (Arrieta et al., 2013b). Similar results were reported by Matsakidou et al. who found that the presence of partially destabilized oil bodies by the unsorbed casein in sodium caseinate film resulted in films with a surface increased hydrophobicity (Matsakidou et al., 2013). However, in CC films the presence of CRV had little influence in the protein-protein bonds since the water contact angle slightly increased with the CRV presence and films remained hydrophilic ( $\theta < 65^\circ$ ). In a previous work it was reported that CRV droplets in SC-CRV films were somewhat smaller than droplets in CC-CRV films (Arrieta et al., 2013b). This result suggests that, in SC-CRV chains, carvacrol molecules did not show surface orientation affecting the film structure and consequently increasing the surface hydrophobicity. In addition, less hydrated  $\text{Na}^+$  ions are expected to displace a lower amount of protons on surface sites (Vogler, 1998). Meanwhile, larger surface protein aggregates, rich in more soluble  $\alpha$ -casein (Srinivasan et al., 1999), were formed in CC-CRV (Arrieta et al., 2013b) where the more strongly hydrated ion  $\text{Ca}^{+2}$  can displace protons on the surface and increase the adhesive molecular forces (Vogler, 1998) showing characteristics of more hydrophilic surface.

### 3.5. Permeation studies

The analysis of the permeability of dye methanolic solutions through the studied films could give some insight on the effect of CRV incorporation on the materials ability to act as an obstacle for the diffusion of compounds with relatively high molar mass, such as most of the common food components (when compared with, e.g.,  $\text{O}_2$ ). Furthermore, dyes are widely used in the food industry, either as colourants (Astray et al., 2011) or as absorbents of waste food products, such as for xenobiotics removal (Safarik et al., 2012). Therefore, crystal violet (CV) was used in this study as an appropriate model to evaluate the possible sorption of those dyes by the studied films. Figure 5a shows the chemical structure of CV at the experimental conditions ( $\text{pH} \approx 6$ ) (Shaw et al., 2003). Table 4 shows the permeability ( $P$ ) and diffusion ( $D_F$ ) coefficients of CV through SC and CC films, while Figure 5b shows a representative caseinate film sample before and after the dye permeation study. Looking to the order of magnitude of the CV diffusion coefficients, it can be concluded that both films are characterized by a moderately hydrophobic character, in agreement with the wettability data. It is also

worth noticing that the incorporation of CVR leads to an increase in time-lag, which can be seen as an induction period in the permeation process. This behaviour can be justified by the increase of interactions between the dye permeant and the polymer matrix (Papancea et al., 2010a) and, consequently, it leads to a decrease in free volume or an increase in the diffusion path. Another interesting result comes out from the comparison of permeability coefficients of the dye through calcium or sodium-based films. The presence of calcium contributed to a large decrease in the dye permeability. These results associated to higher time-lags for CC than for SC can be explained by considering that divalent calcium cations promote cross-linking with protein chains limiting the polymer macromolecules mobility (Arrieta et al., 2013b) and, consequently, dye permeability is reduced. These results are in agreement with OTR.e values. The high affinity of permeants for divalent cations in other biopolymer matrices, such as poly(vinyl alcohol) (PVA), was reported by Papancea et al (Papancea et al., 2010b). Moreover, CC-CRV films were those with the lowest diffusion coefficient showing that carvacrol is contributing to the decrease in the dye concentration gradient and thus difficulting the diffusion process (Valente et al., 2000). This is supported by the calculation of the partition coefficient  $K$  ( $K = P/D_F$ ), which is a measure of the dye concentration in the gel and in the aqueous phase (Cozzolino et al., 2012). In fact, whilst SC films showed a high ability to retain the dye, which is also a value of its affinity for the dye, the ability of CC films for dye retention lead to important decreases in  $K$  up to values lower than 1. Consequently, it can be concluded that the affinity for the dye permeant was higher in the case of SC films.

#### 4. Conclusions

The incorporation of carvacrol into plasticized caseinates was successfully performed to obtain transparent active films. The antimicrobial activity of SC and CC films containing carvacrol was clearly demonstrated against two indicator bacteria. Barrier properties to oxygen were excellent and diffusion of dyes through films was dependent on the caseinate (SC or CC), since CC resulted in less permeable composites due the ability to promote cross-linking. In conclusion, the combination of caseinates plasticized with glycerol and carvacrol as active agent offers high potential for the development of transparent active films for food packaging with excellent barrier properties and antimicrobial activity. Thus, future research in this area should focus on the application of these antimicrobial films to foodstuff. In this sense, studies of antimicrobial effectiveness during the storage and the diffusion of carvacrol to processed food products are currently on-going.

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**Table 1.** Edible films formulations, designations and thickness

Formulation	Sodium Caseinate (wt%)	Calcium Caseinate (wt%)	Glycerol (wt%)	Carvacrol (wt%)	Thickness ( $\mu\text{m}$ )
SC-G25	5	-	25	-	$93 \pm 5$
SC-G25-CRV10	5	-	25	10	$96 \pm 7$
SC-G35	5	-	35	-	$90 \pm 6$
SC-G35-CRV10	5	-	35	10	$91 \pm 4$
CC-G25	-	5	25	-	$108 \pm 10$
CC-G25-CRV10	-	5	25	10	$100 \pm 6$
CC-G35	-	5	35	-	$108 \pm 5$
CC-G35-CRV10	-	5	35	10	$109 \pm 8$

**Table 2.** Colour coordinates from SC and CC edible films

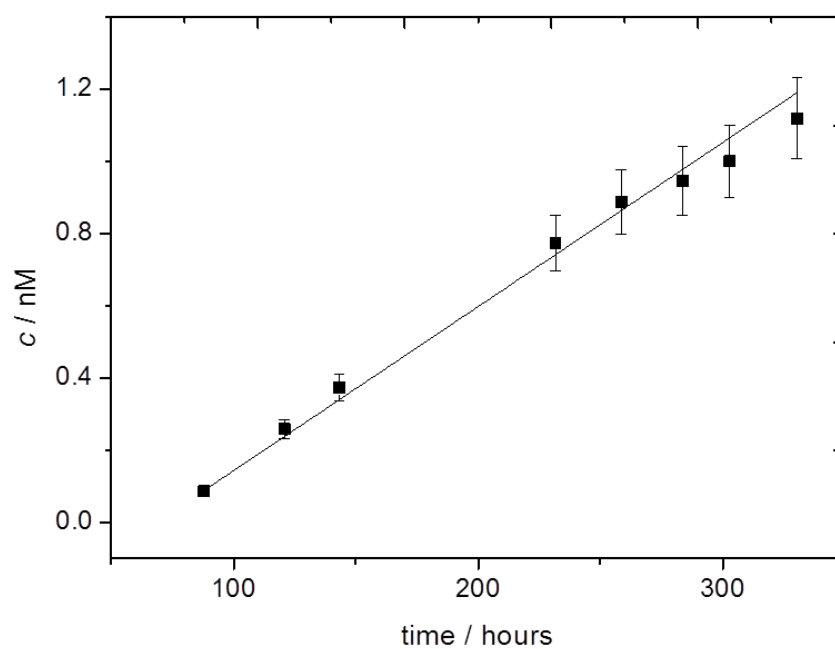
Formulations					
Protein:G:CRV		<i>L</i>	<i>a</i> *	<i>b</i> *	$\Delta E$
SC film	1:0.25:0	92.8 ± 1.3 <sup>a</sup>	-1.6 ± 0.1 <sup>b</sup>	2.8 ± 0.3 <sup>a</sup>	-
	1:0.25:0.1	93.8 ± 0.2 <sup>a</sup>	-1.3 ± 0.1 <sup>a,c</sup>	2.5 ± 0.4 <sup>a,b</sup>	1.2 ± 0.1
	1:0.35:0	93.8 ± 0.1 <sup>a</sup>	-1.4 ± 0.2 <sup>b</sup>	2.6 ± 0.3 <sup>a,b</sup>	-
	1:0.35:0.10	94.0 ± 0.3 <sup>a</sup>	-1.2 ± 0.8 <sup>a,c</sup>	2.2 ± 0.7 <sup>a,b</sup>	0.5 ± 0.1
CC film	1:0.25:0	92.9 ± 0.3 <sup>a</sup>	-1.3 ± 0.1 <sup>a,c</sup>	2.9 ± 0.3 <sup>a</sup>	-
	1:0.25:0.1	92.4 ± 1.1 <sup>b</sup>	-1.4 ± 0.2 <sup>a</sup>	2.7 ± 1.3 <sup>a</sup>	0.5 ± 0.1
	1:0.35:0	93.4 ± 0.1 <sup>a</sup>	-1.3 ± 0.1 <sup>a,c</sup>	2.8 ± 0.1 <sup>a</sup>	-
	1:0.35:0.10	93.5 ± 0.8 <sup>a</sup>	-1.2 ± 0.1 <sup>c</sup>	2.0 ± 0.1 <sup>b</sup>	0.9 ± 0.1

<sup>a-c</sup> Different superscripts within the same column indicate significant differences between formulations ( $P < 0.05$ )

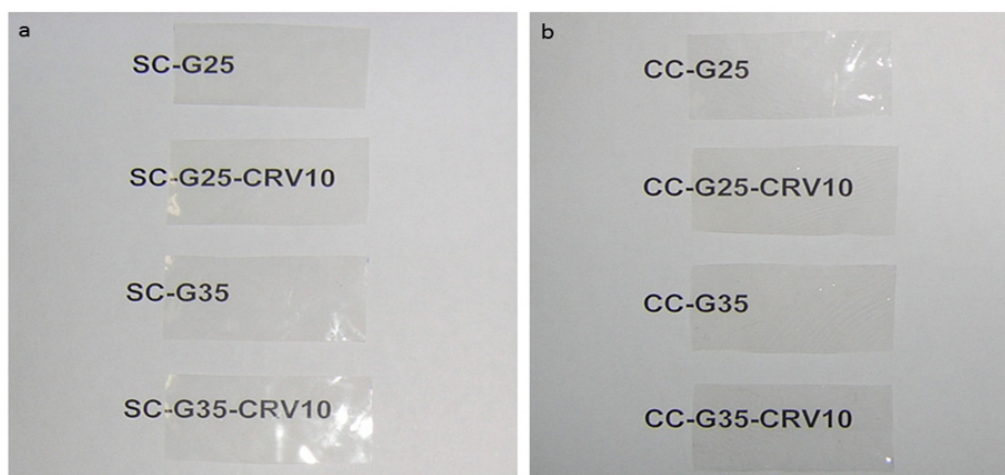
**Table 3.** Wettability of SC and CC edible films

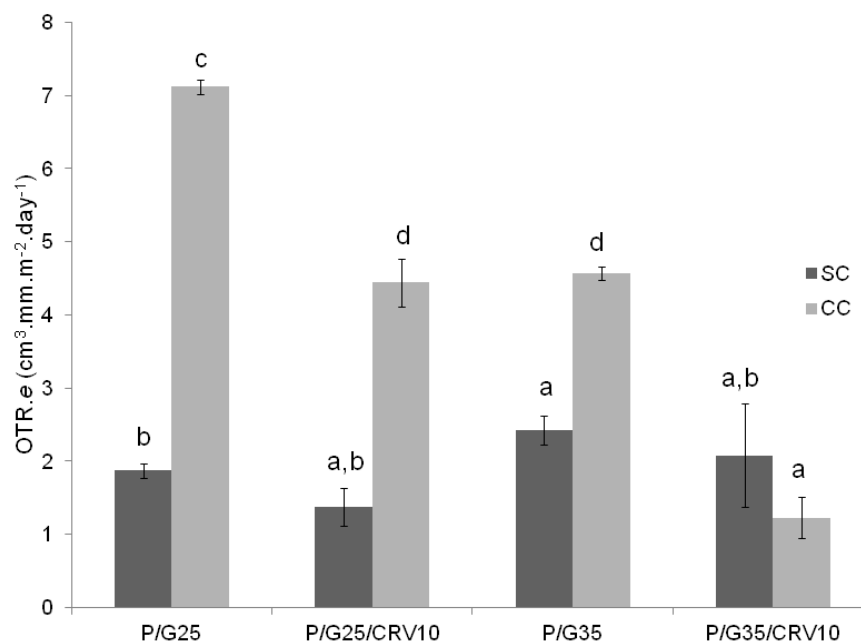
Protein:G:CRV (in weight)	$\theta'$	
	SC	CC
1:0.35:0	56.1 $\pm$ 1.2 <sup>a</sup>	54.0 $\pm$ 2.1 <sup>a</sup>
1:0.35:0.10	83.3 $\pm$ 2.0 <sup>b</sup>	56.5 $\pm$ 0.9 <sup>a</sup>

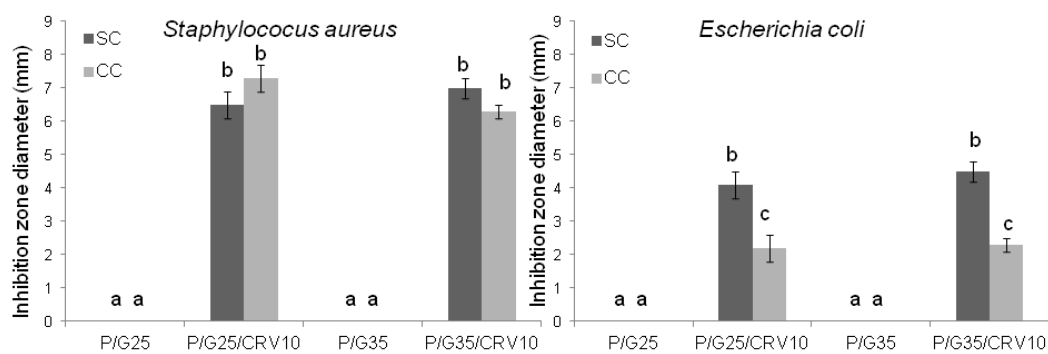
<sup>a-b</sup> Different superscripts within the same column indicate significant differences between formulations (P < 0.05)

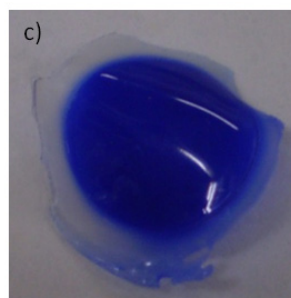
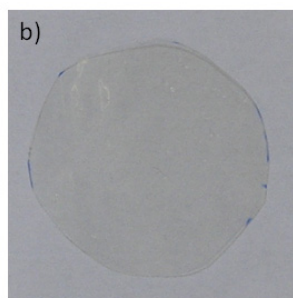
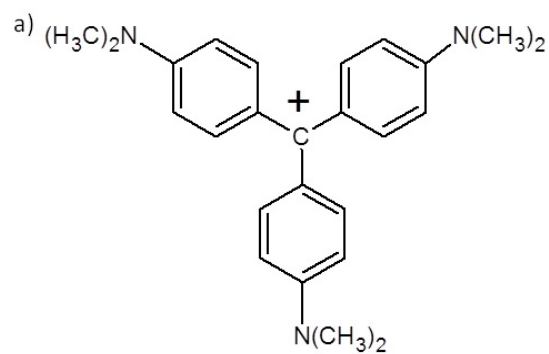












**Highlights**

- Edible active films based on caseinates and carvacrol were prepared
- Carvacrol did not affect the high transparency of caseinates edible films
- Edible films incorporated with carvacrol exhibited antibacterial activity
- Oxygen and dye permeation were lesser in calcium caseinate films due its ability to promote cross-linking